

OPTIMIZATION OF PHYSICO-CHEMICAL PARAMETERS FOR HYPER-PRODUCTION OF LYSINE BY MUTATED STRAIN OF *BREVIBACTERIUM FLAVUM*

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ABSTRACT

Lysine as an essential amino acid supplemented to fulfil the dietary requirement of Poultry feed. In Pakistan all this demand is accomplished by importing lysine for feed sector. Wild *Brevibacterium flavum* was used to induce mutation by random mutagenesis for hyper production of lysine through fermentation. The highest lysine producing strain (BF^{ENU2}) was screened with 21.64 g/L production by mutation treatment of (ENU). It was used for physico- chemical optimization of cultural medium. Molasses with 15 brix having 10- 12% sugar was found optimum for lysine production (25.88g/L) with optimized fermentation period of 48 hours. During optimization of physical parameters significantly (P< 0.05) high yield of lysine 26.69 g/L was produced by 4% inoculum at 30°C and pH 7 with pre- optimized substrate. The optimum concentration of 0.3% NH₄(SO₄)₂ and 0.2% urea was found significant (P< 0.05) with high concentration of Lysine (29.34 g/L) production. The optimum level of CaCO₃ 2% was found statistically (P< 0.05) significant that produced 29.84 g/L of Lysine. The inclusion of optimum soybean hydrolysate (0.5%) and tryptone (0.3%) significantly enhance the titre of Lysine (33.92 g/L). Thus after optimization of various physical and chemical parameters Lysine production enhanced much than wild strain hence these optimum levels can be used for mass production of lysine on pilot scale.

Key- words: Mutated strain; *Brevibacterium flavum*; optimization; physico- chemical; hyper- production

INTRODUCTION

Lysine is cationic in nature with numerous potential uses in agricultural, medicine and pharmaceutical industries. Lysine blocks the bio-availability of arginine required by Herpes virus for reproduction; thus, it can inhibit the symptoms of Chronic Fatigue Dysfunction Syndrome (Ekwealor and Obeta, 2005). It increases body's interferon level and helps in combating infections. In pharmaceutical industries, lysine is used in formulating diets and in amino acid infusion (Nelofer *et al.*, 2008). Lysine demand has steadily increased over the years with an inevitable increase in supply. It is currently being produced worldwide at a rate of 6×10^5 to 8×10^5 tons per year. It is the second largely produced amino acid (Gunji and Yasueda, 2006). At this time, more than 80% of Lysine is manufactured through fermentation of natural materials. The choice of microorganism is very important in the lysine manufacture. Identification of more lysine producing strains, such as strains of *Brevibacterium* and *Corynebacterium* their mutants were developed that are used to enhance the lysine production (Anastassiadis, 2007). Wild strains have ability to synthesize lysine as an essential components but their production is limited. To date, many studies on strain improvement have been carried out using various methods including conventional

mutagenesis, screening, genetic engineering and metabolic engineering (Malothu *et al.*, 2012). Lysine was the first amino acid that was produced with the help of mutant strains that require homoserine for hyper production in industry (Shah and Khan, 2008). Selection of substrate is as important as microbe selection for fermentation (Kothari, 2009). During the selection it is preferred to use cheap and economical substrate such as molasses, cereal bran and starch hydrolysate (Shah and Khan, 2008). With the passage of time to accomplish the increasing demand of lysine manufacturers are trying to optimize numerous levels of carbohydrates and other conditions (Ishikawa *et al.*, 2008). On commercial scale it is manufactured by aspartate kinase resistant mutant of *Corynebacterium glutamicum* obtained by classical screening. Overproduction and secretion carrier of lysine is closely related to each other in an organism. Although secretion carrier of lysine is present in both wild and mutant strains, but over production is shown only by mutant strain (Tryfona and Bustard, 2004). Pakistan is a developing country and imports all the required lysine for food and feed sector. To save the foreign exchange, there is need to develop a complete modal of Lysine production locally. Therefore wild *Brevibacterium flavum* was exposed to random mutation and screened out a mutant strain induced by a chemical mutagen, N- nitroso N- ethyl urea (ENU). Mutant strain (BF^{ENU2}) was further

subjected to optimization of various physical and chemical parameters for hyper- production of lysine.

MATERIALS AND METHODS

Brevibacterium flavum (NRC- 207 F Rev. 2/78) was procured from the International University Tokoyo, Japan. The organism was revived and maintained on nutrient agar plates at pH 7. Analytical grade chemicals (American Sigma Chemicals Company, German company Merck, Across Belgium, BDH of U.K and Fluka of Switzerland) were used during the present work.

Mutagenesis:

Protective ceil of N- nitroso N- ethyl urea (ENU) was carefully opened and various concentrations 100mM, 150mM, 200mM, 250mM, 300mM and 350mM were prepared to make the death curve. Seed culture of 18 hour was freshly prepared by using nutrient broth (NB) medium. Pellet of 1mL parent cells was obtained after 5 min. of centrifugation (10,000 rpm). It was re-suspended in 1mL sodium citrate buffer (pH 4.2) and re- centrifuged for 5 minutes at 10,000 rpm (Javed *et al* 2011). Then pellet obtained was incubated with 1mL optimum concentration of ENU (250mM) for 5, 10, 15, 20, 25 and 30 minutes. During each interval, every concentration of ENU was centrifuged for 5 minutes at 10,000 rpm and then pellet was washed with sodium citrate buffer (pH 4.2) to remove excess of ENU. The pellet thus obtained was re-suspended in NB medium for growth. The growth medium of 100µL was spread on plates of nutrient agar medium with the help of spreader. The plates were incubated for 24 hours at 30°C. Those plates which have 99% kill were further subjected to mutant selection medium and also screened out for more lysine production. For the mutant selection, plates were prepared having minimal medium (MM) for the selection of mutant. The MM was prepared by; glucose 8g, MgSO₄. 7H₂O 0.01g, (NH₄)₂SO₄ 0.2g, NaCl 0.5g, K₂HPO₄ 0.3g, Biotin 5µL, agar 1.5, (1 mM) Homoserine 400 µL/ 20mL, (1 mM) threonine 500 µL/ 20mL, (10 mM) 2-(aminoethyl) L- cysteine (AEC) 200 µL/ 20mL. The colonies were aseptically transferred to the MM plates with and without homoserine, threonine and (AEC) through replica plating method (Rehman *et al.* 2012). The colonies appeared on MM supplemented with 0.1 mM homoserine, 0.1 mM threonine and 10 mM (AEC) and not on without these components were considered as homoserine and threonine auxotroph and resistant to AEC.

Physico-Chemical optimization for Hyper-production of Lysine: During the optimization of (BF^{ENU2}), organism was grown with all the pre-optimized condition of wild *Brevibacterium flavum* (*B. flavum*) for a period of 120 hours to find the optimum incubation period for

maximum lysine production. Different concentrations of cane molasses (10, 15, 20, 25 and 30 brix) were used to optimize the optimum level with mutated BF^{ENU2}. Various degrees of temperature (25, 30, 35, 40°C) were optimized to yield maximum Lysine by using the pre-optimized molasses concentration as a substrate. The effect of various pH (6.6, 6.8, 7, 7.2 and 7.4) were investigated to find the optimum level of pH at which maximum Lysine was secreted. Different percentages (1, 2, 3, 4 and 5%) of inoculum were used to optimize the size of inoculum to get high titre of Lysine (Adnan *et al.*, 2011). Influence of (NH₄)₂SO₄, and urea concentrations (0.1, 0.2, 0.3, 0.4 & 0.5 %) as nitrogen source were investigated to determine the maximum lysine producing level by using (BF^{ENU2}) as fermenting agent with pre-optimized conditions (Sattar *et al.*, 2008). Various concentrations (1, 2, 3, 4 & 5%) of CaCO₃ were analyzed to find the optimum level. Several percentages of soya bean hydrolysate (0.25, 0.5, 0.75 and 1%) and various concentrations of tryptone (0.1, 0.2, 0.3, 0.4 and 0.5%) were used to get the maximum lysine producing concentration by using (BF^{ENU2}) with previously optimized conditions (Athar *et al.*, 2009).

All the parameters were optimized in shake flask of 250 mL (Erlenmeyer flask) having 25 mL total fermentation medium.

Fermentation: The inoculum of mutated *B. flavum* was used for optimization of all the parameters was prepared by following Nasab *et al.* (2007). The optical density of 10- 18 hours culture was adjusted to 0.6 at 600 nm by diluting the culture with sterilized normal saline (Athar *et al.*, 2009). The fermentation medium was prepared by adding optimized concentrations such as molasses (15 brix), MgSO₄.7H₂O (4g), KH₂PO₄.H₂O (2g), NaCl(1g), (NH₄)₂SO₄(3g), CaCO₃(20g), urea (2g), biotin (4mg), soya bean hydrolysate (5mL) and tryptone (3g/L). The pH was adjusted at 7 with 1N NaOH and was autoclaved for 15 minutes. It was cooled at room temperature and inoculated with freshly prepared inoculum. The fermentation was carried out on orbital shaker at 180 rpm at 30°C for 48 hours (Nasab *et al.*, 2007).

The lysine produced was spectrophotometrically estimated from the centrifuged supernatant of fermented broth by following Chaves *et al.* (1988). Processing of data was carried out through a statistical method by using SPSS 13.0 soft-ware (Levesque, 2007).

RESULTS

For hyper production of Lysine, mutated strain was incubated for 120 hours with 4% (10 brix) pre-treated molasses as a substrate and rest of basal medium was same as that used for wild *B. flavum* at 30°C and pH 7. During fermentation lysine production was monitored after every 24 hours. The results showed that significantly

($P < 0.05$) higher yield of Lysine 22.45 g/L after 48 hours and thereafter decline in Lysine titre was observed as shown in (Fig. 1a). From various concentrations (10, 15, 20, 25 & 30) of molasses as substrate 15 brix was found to be optimum used for hyper production of lysine by using mutant *B. flavum*. The highest yield of Lysine 25.88 g/L was found significantly higher ($P < 0.05$) as shown in (Fig. 1b).

Effect of different degree of temperature was investigated and maximum lysine production was observed at 30°C with 15 brix molasses after 48 hours of incubation. Lysine titre was decreased with the rise of temperature. To get the highest growth with good secretion of Lysine, various ranges (6.6, 6.8, 7.0, 7.2 & 7.4) of initial pH of fermentation medium were investigated. Maximum lysine 26.7 g/L was produced at pH 7 with pre- optimized conditions. Different sizes of inoculum (1, 2, 3, 4 & 5) were used to find the optimum inoculum size for hyper production of Lysine from mutant *B. flavum* with 15 brix molasses as substrate for 48 hours of fermentation period at 30°C and pH 7. The trend of produced Lysine showed that significantly ($P < 0.05$) higher titre 27.84 g/L of Lysine was investigated with 4% of inoculum size given in (table 1).

Different concentrations (0.1, 0.2, 0.3, 0.4 & 0.5%) of $\text{NH}_4(\text{SO}_4)_2$ and urea as nitrogen source were investigated. The highest yield of lysine (28.86 g/L) was obtained with 0.3% concentration of $\text{NH}_4(\text{SO}_4)_2$ and significantly ($P < 0.05$) high titre of Lysine (29.34 g/L) was obtained with 0.2% urea with all the other pre- optimized conditions as shown in (Fig. 2a & b). From the numerous concentrations (1, 2, 3, 4 & 5%) of CaCO_3 2% resulted in maximum production of lysine 29.84 g/L with pre- optimized concentrations as shown in fig. 3.

Various concentrations (0.25, 0.5, 0.75 & 1%) of soybean hydrolysate were examined with pre-optimized fermentation medium for hyper production of Lysine. The significantly ($P < 0.05$) higher yield (31.2 g/L) of Lysine was achieved with 0.5% soybean hydrolysate shown in (Fig. 4a). The inclusion of various concentrations (0.1, 0.2, 0.3, 0.4 & 0.5 %) of tryptone for hyper production of Lysine were tested by using 4% inoculum of mutant *B. flavum* as fermenting agent as shown in (fig. 4b). The high titre 33.92 g/L of Lysine was estimated with 0.3% Tryptone and was found statistically significant ($P < 0.05$).

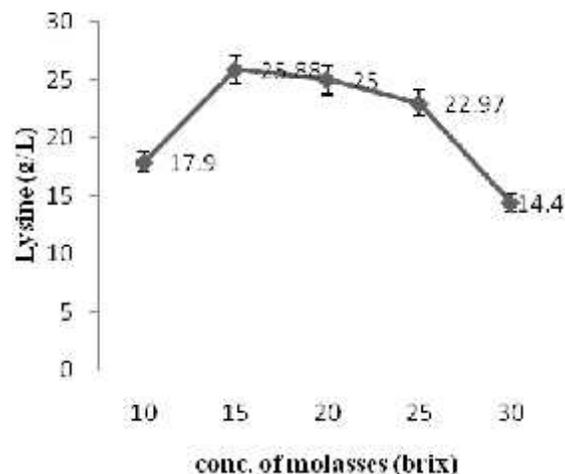
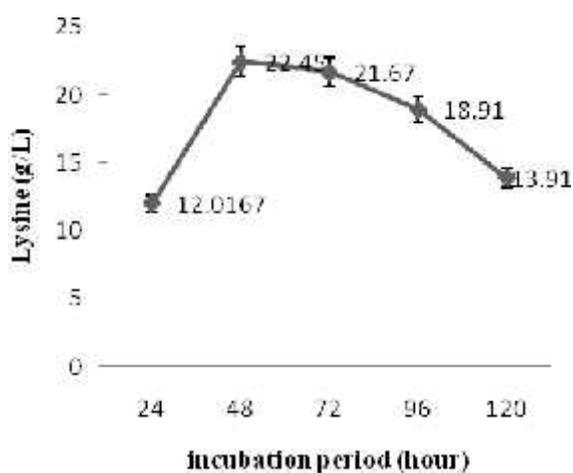


Figure 1 (a): Optimization of incubation period for the production of Lysine from mutant *B. flavum* with 4% (10 brix) molasses as a substrate.

Figure 1 (b): Effect of different conc. of molasses (brix) for the production of Lysine by (BF^{ENU2}) at 30°C and 48 hours of incubation time.

Table 1. Effect of different physical parameters on Lysine production during fermentation by mutant BF^{ENU2}

Temperature (°C)		pH		Inoculum size (%)	
Level	mean± SE	Level	mean± SE	level	mean± SE
25	8.85± 0.28	6.6	10.47± 0.17	1	11.96± 0.28
30	26.43± 0.08*	6.8	22.21± 0.16	2	17.55± 0.42
35	18.20± 0.15	7.0	26.69± 0.08*	3	26.19± 0.27
40	9.04± 0.03	7.2	25.39± 0.03	4	27.84± 0.54*
-	-	7.4	24.85± 0.03	5	23.22± 0.27

Each value is the mean of three replicates, ± SE represented standard error among different levels, numbers followed by superscript * showed significant level ($P < 0.05$) of Lysine produced with 15 brix molasses, 180 rpm and after 48 hours of incubation.

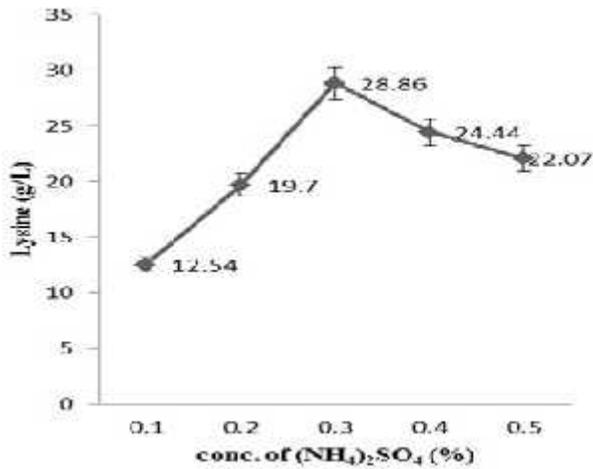


Figure 2 (a): Influence of various concentrations of $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source for hyper production of Lysine from 4% inoculum of $(\text{BF}^{\text{ENU}2})$ with 15 brix molasses.

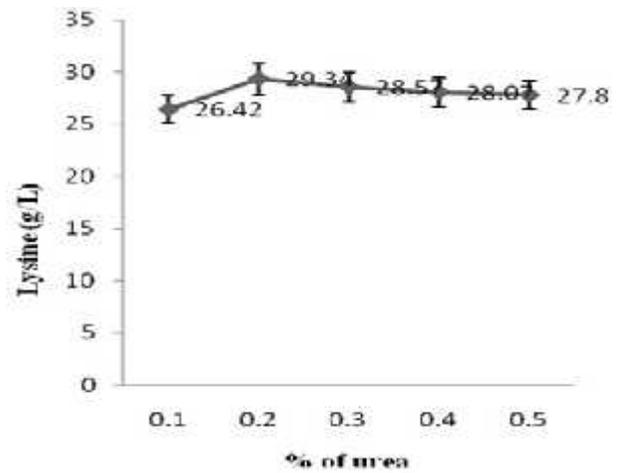


Figure 2 (b): Effect of various concentrations of urea as organic nitrogen source for hyper production of Lysine from 4% inoculum of $(\text{BF}^{\text{ENU}2})$ with 15 brix molasses.

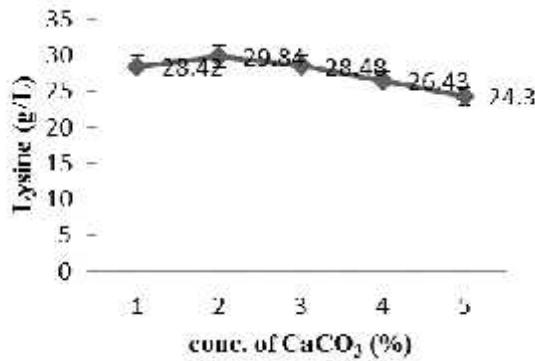


Figure 3: Effect of various concentrations of CaCO_3 for hyper production of Lysine from 4% inoculum of $(\text{BF}^{\text{ENU}2})$ with 15 brix molasses

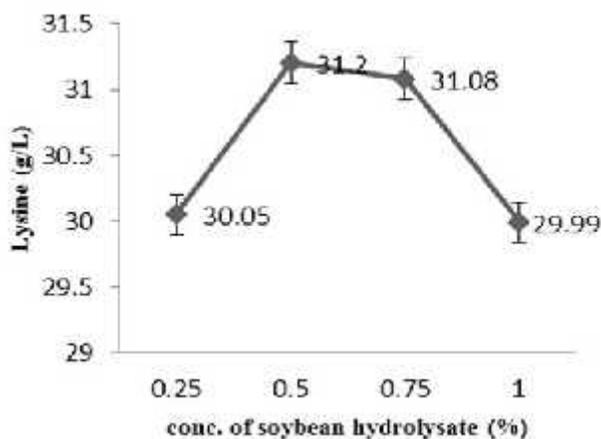


Figure 4 (a): Influence of various concentrations of soybean hydrolysate for hyper production of Lysine from 4% inoculum of $(\text{BF}^{\text{ENU}2})$ with 15 brix molasses as substrate

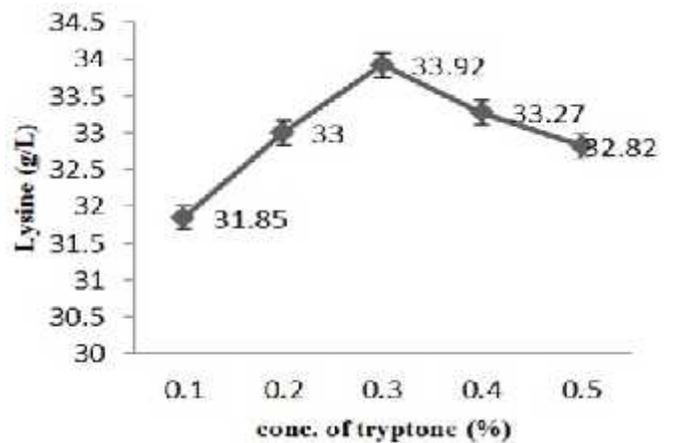


Figure 4 (b): Effect of various concentrations of tryptone for hyper production of Lysine from 4% inoculum of $(\text{BF}^{\text{ENU}2})$ with pre- optimized culturing conditions

DISCUSSION

Lysine required for poultry feed is imported in Pakistan to fulfil the desired dietary needs. Present study was designed to produce maximum lysine by utilizing cheap source to save the foreign exchange. To produce lysine locally mutant strain was developed by using ENU as a chemical mutagen from wild *B. flavum* that was capable to produce more lysine. Moreover, different Physico- Chemical parameters were optimized to have hyper- production of Lysine through fermentation by using mutated *B. flavum* (BF^{ENU2}). After investigation of different parameters it was concluded that mutated strain was better than wild strain in terms of more Lysine producer in less incubation period. Moreover this strain would be industrially more attractive and economical as it produced maximum Lysine in less fermentation time. To optimize other parameter this incubation period of 48 hours was used to get the highest titre of Lysine. Hence after incubation time study it was revealed that mutated strain showed comparatively high metabolic activity and early decline in growth phase. Whereas wild *B. flavum* showed maximum Lysine titre (9.1 g/L) after 70 hours and afterwards its yield dropped. The decline of Lysine titre could be associated with the decline of bacterial growth or depletion of nutrients from the fermentation medium. Therefore optimum fermentation period for mutant and wild strain was 48 and 72 hours respectively. The present results of incubation period are coincided with Mumtaz *et al.* (2000) that reported 48 hours of incubation for fermentation to enhance Lysine contents (189mg/ 100mL) of distillery sludge by using *B. flavum* as fermenting agent. Similarly, Naz *et al.* (2001) reported 48 hours as optimum fermentation period for maximum lysine production 21.48 mg/g on the basis of protein from UV- mutated *B. flavum*. Adnan *et al.* (2011) optimized incubation time of 48 hours for enhanced Lysine 23.57g/kg by solid substrate fermentation with *Brevibacterium linens*. While Rehman *et al.* (2012) investigated maximum Lysine production (18mg/ 1mL) by UV- mutated *C. glutamicum* after 60 hours of incubation. Metabolic flux was demonstrated by Wittmann and Becker (2007) after mutation. Three strains of *E. coli* were developed two strains of them produced high Lysine (14.97 & 13.39 g/L) after 72 hours of incubation and third manufactured maximum lysine (13.88 g/L) after 48 hours of incubation. The results of Javed *et al.* (2011) were different from present study. Mutant strain of *B. flavum* was developed by using mutagen ENU and cultured organism to optimize fermentation period and reported the highest yield 67.8 g/L after 24 hours. Ali *et al.* (2009) demonstrated fermentation of yeast sludge with mutated *B. flavum* AJ/SA-315 enhanced the lysine content from 1.59% to 3.71% with 72 hours of incubation period.

The substrate is the most important and basic requirement for microbial biosynthesis. A cheap substrate with multiple nutrients other than as a good carbon source is ideal for any biotechnological process. Molasses proved to be a best agricultural by-product that used in present study as good carbon source. Therefore, 15 brix of molasses were used as optimum level in fermentation medium by using mutant *B. flavum* as fermenting agent. As the concentration of molasses increased beyond 15 brix, the titre of Lysine decreased that could be due to the inhibitory effect of increased sugar contents on lysine synthesis. Hydrolysed and un-hydrolysed sugar contents (34% and 32% respectively) of pre-treated molasses were estimated. That showed sugar content of 10- 12% in 15 brix molasses were optimum level for hyper production of molasses. Industrially most favourable cheap substrate that was used for amino acid fermentation is molasses (Ikeda, 2003). Molasses contain sucrose as main content and other sugars as minor contents are glucose, fructose, raffinose and some oligo or polysaccharides. Minerals found in molasses are potassium, sodium, calcium and magnesium. Moreover amino acids, vitamins and no fats contents made it an attractive cheap substrate (Chatterjee, 1998). Temperature influences the growth and metabolites production of an organism, while optimization same temperature 30°C was found optimum for high Lysine production by mutated and wild *B. flavum*. This temperature was also favourable for the maximum growth of *B. flavum* as coryneform bacteria grow well between 25- 40°C (Anastassiadis, 2007). The above results are in line with Javed *et al.* (2011) that observed high titre of Lysine at 30°C and justified that *B. flavum* as a mesophilic that grows with high enzyme activity at moderate temperature 20- 40°C. The results of other researcher are also in line with the present studies, they investigated maximum Lysine production at 30°C during batch fermentation (Tada *et al.*, 2000; Ohnishi *et al.*, 2005; Gerovaet *et al.*, 2011). While Ali *et al.* (2009) reported 37°C as optimum temperature and Javaidet *et al.* (2012) developed a UV mutant *B. flavum* that produced high Lysine at 32 °C.

The produced metabolites also affect the pH of the medium and growth of the microbes, therefore the final pH of fermented broth at the end of fermentation was also examined. Maintenance of pH between 6.9- 7.0 during the fermentation produced highest lysine titre in the fermented medium. The results of present studies are in line with Javed *et al.* (2011) that the maximum Lysine production was (69.5 g/L) at pH 7 in growth medium as compared to 58.1, 65.9 and 34.1 g/L lysine at pH 6.5, 7.5 and 8.0 respectively. Similar results were reported by Sattar *et al.* (2008) that high titre of Lysine was obtained 34mg/ 100mL at pH 7 after 48 hours of incubation. The results of Naz *et al.* (2001) were also in agreement with present study. While Rehman *et al.* (2012) investigated 12.5 g/L of lysine at optimum value of pH 7.6 after 60

hours of incubation. Vegetative growth of bacterium may be promoted at the cost of lysine production due to competition for available nutrient (Reddy *et al.*, 2008). Therefore, exponentially growing mutated *B. flavum* inoculum of 8 hours was used for hyper production of Lysine having 0.6 OD at 600nm. While wild strain showed 0.6 OD at 600 nm after 18 hours that showed the enhanced growth rate of mutated strain with early stationary phase as compared to wild *B. flavum*. The present result of inoculum size was in agreement with Adnan *et al.* (2011) whereas Javaid *et al.* (2012) and Rehman *et al.* (2012) reported maximum lysine production with 8% and 10% inoculum size respectively for high lysine production.

Since two amino groups (-NH₂) are present in lysine molecule, therefore a constant supply of nitrogen is required for the maximum Lysine biosynthesis. Different sources of nitrogen like NH₄NO₃, NH₄(SO₄)₂ and urea were tried for the production of lysine and NH₄(SO₄)₂ was found the best source for wild (*B. flavum*). The high concentration 0.3% of NH₄(SO₄)₂ could be attributed with the increased demand of nitrogen for high Lysine production by mutated *B. flavum*. Urea being a neutral organic nitrogen source was also used for hyper production of Lysine. The lysine production was significantly (P< 0.05) enhanced up to (29.34 g/L) with optimum level of urea (0.2%). Whereas Rehman *et al.*, (2012) revealed the optimum concentration of NH₄(SO₄)₂ 3 and 3.5% for lysine production from various basal media (FM3 and FM1 respectively). Although Javaid *et al.* (2012) reported 0.1 % NH₄(SO₄)₂ for maximum yield of lysine 8.8 g/L. The use of different combinations of inorganic and organic nitrogen sources with various levels might be the reason of difference in optimum concentrations of their findings with the present study. As NH₄(SO₄)₂ was found to be a best inorganic nitrogen source, urea also proved to be a good organic ammonia supplier for the maximum production of lysine. Similarly the results of Sattar *et al.* (2008) and Ali *et al.* (2009) are also in agreement with the present finding and confirmed that urea; a low cost neutral fertilizer that does not increase the pH of fermentation medium, supported maximum Lysine production.

CaCO₃ was added in the fermentation medium not only as calcium source but also as a buffer (Anastassiadis, 2007). From the numerous concentrations of CaCO₃ 2% resulted in maximum production of lysine with 15 brix molasses as substrate. Whereas CaCO₃ (4%) was found optimum for hyper production of Lysine (19.01 g/L) with wild *B. flavum*. Furthermore 2% CaCO₃ was completely assimilated at the end of fermentation with maximum titre of lysine. Same concentration of CaCO₃ was described by Shah *et al.* (2002) and Nelofer *et al.* (2007) in their fermentative production of lysine. Whereas Rehman *et al.* (2012) determined the maximum lysine (17.5 mg/mL) with 0.2% of CaCO₃. Many

industrial by products like cane molasses, corn starch hydrolysate, corn steep liquor, soybean meal and soybean hydrolysate can be exploited not only as carbon and nitrogen sources but it contains minerals and some amino acids in fermentation medium for large scale production of lysine and thus fermented broth rich in lysine proved to be useful for broiler feed. Moreover the presence of some essential nutrients required by the mutant for its growth make it more attractive to use with optimum level. That is the reason soybean hydrolysate was prepared and significantly (P< 0.05) higher yield (31.2 g/L) of Lysine was achieved with 0.5% soybean hydrolysate. These results supported the findings of Rehman *et al.* (2012) and Adnan *et al.* (2011) that inclusion of these hydrolysates fruitfully enhanced the lysine titre. Sattar *et al.* (2008) also added different percentages of protein hydrolysate of sesame meal to improve the lysine production. Tryptone is prepared by an enzymatic digestion of casein which serves as peptone and tryptophan rich source for fermentation (Sezonov *et al.*, 2007). The high titre 33.92 g/L of Lysine was estimated with 0.3% Tryptone and was found statistically significant at a level of (P< 0.05). Tryptone 0.05% was also found as a significant nitrogen source by Sezonov *et al.* (2007) for hyper production of α -amylase by *Bacillus subtilis* (DJ5). The over expression of aspartokinase in *C. glutamicum* was investigated by Rastegari *et al.* (2013), used LB medium in that study. It was reported that LB medium included with 1% bacterial tryptone, 5% yeast extract and 5% NaCl.

Conclusion: All the physico- chemical parameters were optimized that produced maximum Lysine 33.92 g/L on micro- scale in 250 mL Erlenmeyer flask by mutated *B. flavum* much higher than wild *B. flavum*. These optimized cultural conditions will be further subjected for the mass production of Lysine in 7.5 L fermenter.

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